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Mitosis is the process by which cells faithfully segregate their genetic material. This process is carried out by the mitotic spindle, which consists of a dynamic array of microtubules responsible for distributing replicated chromatids to each daughter cell. This proposal focuses on XKCM1, a kinesin-related protein required for mitotic spindle assembly *in vitro*. Our previous results indicated that XKCM1 acts by controlling the polymerization dynamics of microtubules which are necessary for spindle assembly. We have carried out a detailed mechanistic biochemistry study which showed that XKCM1 acts by binding to microtubules, causing a conformational change of the microtubule that results in microtubule depolymerization. This was a very novel and unexpected finding and has strong implications in understanding the biochemical mechansims of this class of protein as well as understanding the role of microtubule dynamics in cellular fuction. We have shown that inhibition of a specific pool of XKCM1, that which is bound to kinetochores, causes a misalignment of chromosomes on the spindle. This data provides the first molecular handle on a protein that can couple microtubule dynamics to chromosome movement. The analysis of its function in live cells will be an important area of future research.

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Claire E Walczak 5/6/98

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## XKCM1: A KINESIN-RELATED PROTEIN REQUIRED FOR MITOTIC SPINDLE ASSEMBLY

### INTRODUCTION:

The faithful segregation of genetic material to daughter cells which occurs during mitosis is essential for the survival of an organism. This process is carried out by the mitotic spindle, which consists of a dynamic array of microtubules responsible for distributing replicated sister chromatids to each daughter cell.

Microtubules are polar polymers that exhibit nonequillibrium polymerization dynamics termed dynamic instability (Mitchison and Kirschner, 1984). Microtubules can coexist in both growing and shrinking states; these states interconvert frequently and stochastically both *in vitro* and *in vivo*. Microtubules undergo periods of growth and shrinkage interspersed with stochastic transitions between these states called catastrophe (change from growth to shrinkage) and rescue (change from shrinkage to growth). Dynamics microtubules may be important in chromosome movement by pushing and pulling on the kinetochore, the specialized site where microtubules contact mitotic chromosomes. In cells microtubule turnover increases dramatically during mitosis relative to interphase and probably plays a crucial role in the assembly of the mitotic spindle (reviewed in (Inoué and Salmon, 1995)). An analysis of microtubule behavior in *Xenopus* egg extracts showed that the polymerization dynamics of microtubules *in vivo* are significantly different from that of pure tubulin, suggesting the presence of cellular factors that regulate dynamics (Belmont et al., 1990; Verde et al., 1990; Verde et al., 1992). These studies also showed that mitotic microtubules are much more dynamic than interphase microtubules, suggesting that there exist one or more cell-cycle regulated factors that affect microtubule dynamics.

During mitosis, mechanical force is essential to separate spindle poles, to bring chromosomes to the equatorial plate at metaphase, to maintain the intact spindle, and to drive the separation of sister chromatids at anaphase. Many of these processes are likely to involve the use of microtubule-based motor proteins which couple the energy of ATP hydrolysis to force production and translocation along the microtubule (reviewed in (Sawin and Endow, 1993; Bloom and Endow, 1995; Vernos and Karsenti, 1996)). Kinesin is the originally identified member of a family of proteins, called kinesin-related proteins (KRPs), which have high sequence similarity to the motor domain of kinesin. Several KRPs are implicated in the assembly and function of mitotic and meiotic spindles (reviewed in (Sawin and Endow, 1993; Vernos and Karsenti, 1996)).

*Xenopus* laevis provides an excellent system in which to study the role of KRPs in mitosis. Mitotic spindles can be assembled *in vitro* in egg extracts (Sawin and Mitchison, 1991), and anaphase chromosome movement can also be observed (Shamu and Murray, 1992). Immunodepletion and antibody inhibition of proteins from egg extracts has been used to determine the effect of loss of a motor protein on spindle morphology and function (Sawin et al., 1992; Vernos et al., 1995; Boleti et al., 1996). Spindles assembled *in vitro* are well-suited for microscopic analysis, and the detailed dynamic behavior of microtubules can be quantitated using fluorescent probes (Sawin and Mitchison, 1991). In addition, one can study cell cycle regulation of motor function, which is likely to be required to ensure forces are produced at the proper time during mitosis.

To explore further the mechanisms of mitotic spindle assembly and motor protein function, I isolated KRPs from *Xenopus* that might be important in this process. I used an antibody to a conserved region of the kinesin motor domain (Sawin et al., 1992) to isolate clones encoding KRPs from a *Xenopus* ovary cDNA library. One of the identified KRPs, which I named XKCM1 (for *Xenopus* Kinesin Central Motor 1) is essential for mitotic spindle assembly *in vitro*, localizes to centromeres, and regulates the polymerization dynamics of microtubules (Walczak et al., 1996). This proposal focuses on the further study

of the *in vivo* function of XKCM1, its cell cycle regulation, and the beginnings of an analysis of its structure.

**BODY:**

**Experimental Methods, Assumptions, and Procedures:**

Protein Expression and Purification: Domains of the XKCM1 gene were subcloned into an expression vector that fuses glutathione-S-transferase (GST) to the amino terminus of the protein. Amino acids 2-263 (NT), amino acids 267-592 (CD), and amino acids 593-730 (CT) were expressed as GST-fusion proteins in bacteria and purified on glutathione agarose. The purified proteins were dialyzed, sucrose was added to 10% for stability, and they were frozen in liquid nitrogen and stored at -80 °C until use.

The full-length XKCM1 protein was not active when produced in bacteria so it was expressed in insect Sf-9 cells using the baculovirus expression system. Insect Sf-9 cells were cotransfected with a transfer vector containing the full length XKCM1 gene and BaculoGold virus DNA (Pharmingen) using calcium-phosphate precipitation. Transfection supernatants were plaque purified, and baculovirus stocks were amplified and used for infections. Protein was isolated from 250 ml of cells per prep. The cells were lysed by freeze-thaw and much of the XKCM1 was found in the soluble pool. The XKCM1 protein was purified to homogeneity by a combination of SP-sepharose cation exchange chromatography, gel filtration chromatography and a high resolution Mono-S column. The peak fractions were pooled, sucrose was added to 10% w/v, and then the samples were quick frozen in liquid nitrogen and stored at -80 °C. Using this procedure, we are routinely able to obtain greater than 1 mg of protein, and the protein maintains activity for at least 6 months when stored frozen.

Assays for XKCM1 Activity: Two alternative assays are used to detect XKCM1 activity. The first assay is a simple microscopy assay. We make fluorescently labeled taxol-stabilized microtubules mix them with XKCM1 and ATP and visualize the depolymerization of the fluorescent microtubules by microscopy. This assay is generally used as a qualitative assay to detect the relative activity of different XKCM1 samples. The second assay involves sedimentation of microtubules. Microtubules are large polymers that readily sediment when centrifuged under certain conditions whereas unpolymerized tubulin dimer will not sediment. When XKCM1 an ATP are added to microtubule polymer, the depolymerization caused by XKCM1 activity release the microtubule polymer into the supernatant. The amount of soluble tubulin released can readily be quantitated under various conditions.

Cell Culture and Microinjection: For all injection studies, PtK2 cells were used. Cells were grown on polylysine coated coverslips at 37 °C to sub-confluency and generally used at approximately 36 hrs after plating. This resulted in a culture with a mitotic index sufficiently high to be able to readily find mitotic cells for injection. Cells on coverslips were transferred to the microscope stage in a special holder which maintained the temperature at 37 °C during the course of the experiment. Cells were microinjected with either control antibody or anti-XKCM1 antibodies and followed for 1-24 hours by time-lapse video microscopy . The time that it took to traverse mitosis was analyzed. Alternatively, a field of cells was injected and then fixed after 24 hrs, stained with an antibody to visualize the microtubules and then analyzed.

Spindle Assembly: Cytostatic factor-arrested extracts were prepared essentially as described (Murray, 1991) except that the crushing spin was performed at 10,000 rpm, 15', full brake in an SW55 Ti rotor (Beckman). Mitotic spindle assembly was carried out as described (Sawin

and Mitchison, 1991), using only freshly prepared extracts. The final concentration of sperm nuclei was 150 sperm/μl. For fusion protein addition experiments and for immunofluorescent staining of spindles, CSF extracts were cycled into interphase with the addition of $Ca^{2+}$ and then back into mitosis with fresh CSF extract as described (Sawin and Mitchison, 1991; Shamu and Murray, 1992).

Phosphorylation Experiments: Fusion proteins were phosphorylated by incubation with purified kinases and gamma $^{32}P$- ATP and then run on polyacrylamide gels. Phosphate incorporation was detected by autoradiography. Alternatively, labeled ATP was added to an extract +/- fusion protein, and the endogenous protein or the fusion protein was isolated by immunoprecipitation onto beads. Phosphate incorporation was determined as above.

**Results and Discussion**

XKCM1 is Required for Spindle Assembly in vitro: I have been focusing my studies a novel KRP called XKCM1 for Xenopus Kinesin Central Motor 1. XKCM1 is an 85 kDa protein with an N-terminal globular domain (NT), a central kinesin-like catalytic domain (CD), and a short C-terminal tail (CT). Antibodies against XKCM1 stain mitotic kinetochores and spindle poles in tissue culture cells. These antibodies were used in immunodepletion and antibody addition experiments which showed that XKCM1 is required for both establishment and maintenance of mitotic spindles (Fig. 1).

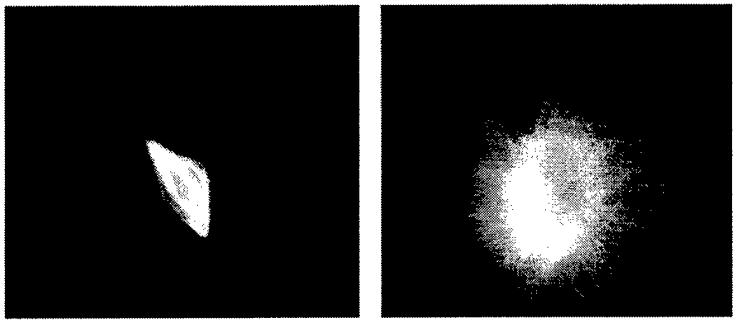


Figure 1 Under control conditions in the presence of a non-immune IgG, normal bipolar mitotic spindles are formed. In the presence of anti-XKCM1 antibodies, huge microtubule asters are formed that contain more numerous and abnormally long microtubules. These large asters arise because XKCM1 is essential to depolymerize microtubules during spindle assembly.

In the absence of XKCM1 function, large microtubule asters are formed that have both longer and more numerous microtubules. This long microtubule defect can be rescued by the addition of purified XKCM1 protein suggesting that these results are due specifically to loss of XKCM1 and not to loss of some other protein. The extremely long microtubules seen in the XKCM1-depleted structures suggested that some aspect of microtubule dynamics was altered in the absence of XKCM1 function. To test this possibility, we measured the parameters of dynamic instability in extracts that lacked XKCM1 activity. We found that the microtubules behaved normally in the absence of XKCM1 function except that they transited from growth to shrinkage less frequently (catastrophe frequency) suggesting that XKCM1 itself may be promoting microtubule depolymerization. This was a novel and unexpected finding for a kinesin-related protein.

Pure XKCM1 Can Depolymerize Microtubules: Our previous results in extracts implied that XKCM1 itself could act to destabilize microtubules. We tested this idea by expressing recombinant XKCM1 in insect Sf-9 cells and looking at its ability to act on pure microtubule substrates. We found that purified XKCM1 protein can destabilize pure microtubules in an ATP-dependent manner. This suggests that XKCM1 is using its conserved kinesin-like ATPase domain to destabilize microtubules (Fig. 2).

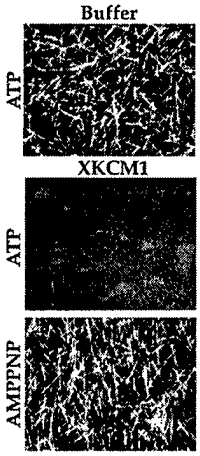


Fig. 2. Rhodamine-lablelled microtubules were polymerized with the non-hyrolyzable GTP analog GMP-CPP to make fluorescent microtubules. When incubated with buffer, you see a field of microtubules. Incubation of the microtubules with XKCM1 and ATP causes total depolymerization of the microtubules. This activity is completely inhibited when the non-hydrolyzable ATP analog AMP-PNP is used. This establishes that XKCM1 is an ATP-dependent microtubule destabilizing factor.

An analysis of different types of microtubule substrates showed that XKCM1 activity was potent, and it was capable of inducing depolymerization of both taxol and GMP-CPP microtubules (GMP-CPP microtubules are polymerized in the presence of a non-hydrolyzable GTP analog and are generally very stable). XKCM1 could induce complete depolymerization of these stabilized microtubules in 15' at a ratio of 1 XKCM1 molecule to 200 tubulin dimers, suggesting a catalytic activity of XKCM1.

Because XKCM1 was a member of the kinesin-related protein family, we expected that XKCM1 was a motor protein that could walk along a microtubule substrate and then induce microtubule depolymerization only at the end of the microtubule. All attempts at generating XKCM1-induced microtubule motility failed although the protein preps did have ATPase activity and were capable of inducing microtubule-depolymerization in solution. Furthermore we noticed in the course of attempting the motility assays, that XKCM1 bound to the glass surface of the microscope slide was still able to induce microtubule depolymerization of the surface-bound microtubules. This suggests that the XKCM1 bound to the glass surface is still active and not just denatured by the glass surface. If XKCM1 were indeed a motor, then we would predict that the depolymerization of the microtubule would be polar (as all motors exhibit known directionality), and would only occur from one end of the microtubule. We found however that XKCM1 induced depolymerization from both ends of the microtubule, consistent with our idea that XKCM1 is not a conventional member of the KRP family but rather has used the general motor mechanism to induce depolymerization of microtubules rather than translocation along its surface (Fig. 3).

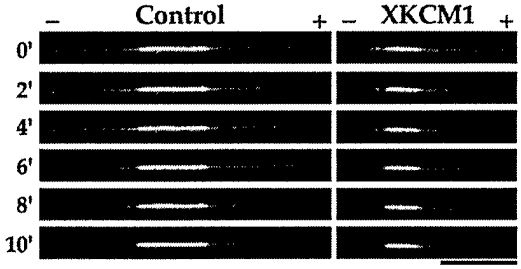


Fig. 3. Polarity marked microtubules were coated on a glass microscope slide. Incubation with a control buffer had no effect. However incubation with XKCM1 and ATP allowed for microtubule depolymerization from both ends of the microtubule. This establishes that XKCM1 is non-polar in its activity.

A further analysis of the mechanism of XKCM1-induced microtubule depolymerization revealed that XKCM1 binding to the end of a microtubule causes a conformational change in the microtubule which puts the microtubule in an unstable conformation (Fig. 4).

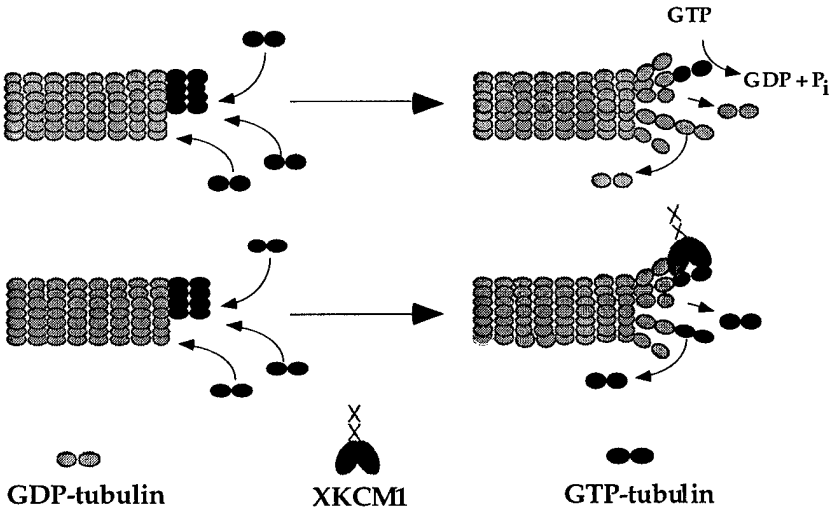


Figure 4. In a control solution of tubulin (top), microtubules are polymerized by the addition of GTP-tubulin to the end of the polymer. At some frequency, the GTP is hydrolyzed causing a conformation change in the microtubule that favors depolymerization. In the presence of XKCM1 protein (bottom), the XKCM1 binds to the GTP-tubulin subunits inducing a conformational change in the microtubule which mimics GTP hydrolysis and enhances depolymerization of the microtubule

This unstable conformation is unfavorable for microtubule stability and allows for microtubule depolymerization to occur. ATP hydrolysis appears to be required to recycle the XKCM1 protein for subsequent rounds of microtubule depolymerization. The activities of XKCM1 are unique for a kinesin-related protein and are valuable for understanding the biochemical mechanism of motor proteins. Furthermore, our results strength the importance of determining the 3-dimensional structure of this protein using X-ray crystallography because of the differences in activity between XKCM1 and other members of the kinesin-related protein family.

XKCM1 Function at Kinetochores is Required for Chromosome Alignment: The ability of XKCM1 to depolymerize microtubules and its requirement for spindle assembly in extracts is presumably carried out by the soluble pool of the protein. This soluble pool of the protein can bind to and release from microtubules and regulate the bulk polymerization dynamics of cytoplasmic and spindle microtubules in cells. The control of the bulk polymerization dynamics of cellular microtubules will be important for cell function and for spindle assembly. In addition, a portion of XKCM1 is specifically bound to kinetochores and may be important in chromosome movement by controlling the local polymerization dynamics of kinetochore-bound microtubules.

We wanted to be able to dissect the different roles of XKCM1 in cells to be able to explore its molecular function in different microtubule-based processes. To begin to address this problem, we sought a reagent that could specifically inhibit only one pool of the XKCM1 protein- that which was specifically bound to kinetochores. We rationalized that if we could identify the kinetochore binding site of XKCM1, we could generate a dominant-negative form of the protein which when added to cells or extracts would bind to kinetochores and compete off the full-length functional XKCM1 protein. We generated glutathione-S-transferase (GST) fusion proteins to the N-terminal globular domain (GST-NT), the centrally located catalytic domain (GST-CD), and the C-terminal alpha-helical tail (GST-CT). These were expressed in bacteria, purified, and then added to extracts prior to spindle assembly. The GST-CT construct had no effect when added to extracts or to pure microtubules. A biochemical analysis revealed that this domain is responsible for dimerization of the XKCM1. The GST-CD domain had no effects when added to extracts. With pure microtubules, it was not active at inducing microtubule depolymerization as was the full length protein, but this domain was sufficient to bind to microtubules suggesting that the kinesin-like microtubule binding site is still functional. When added to extracts, the GST-NT protein targeted to kinetochores during spindle assembly suggesting that the N-terminal domain of XKCM1 is sufficient for kinetochore localization (Fig. 5).

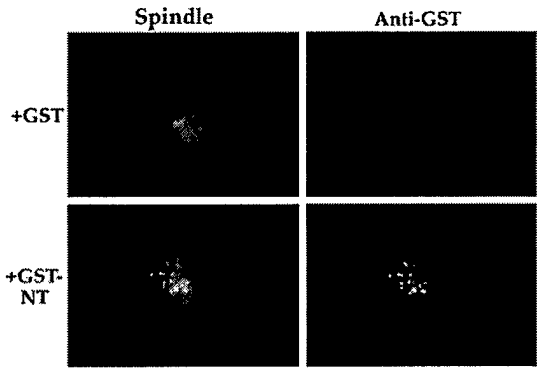


Fig. 5. Spindles were assembled in Xenopus egg extracts with either a control GST addition or a GST-NT addition. The NT of XKCM1 was sufficient to target to kinetochores as detected by anti-GST staining.

The displacement of endogenous XKCM1 from kinetochores by GST-NT caused a misalignment of chromosomes on the metaphase plate and an increase in astral microtubules without affecting the global spindle structure. These results suggest that XKCM1 is important in chromosome positioning within the spindle. Because this reagent allowed spindle formation in these extracts, this suggests that the endogenous soluble pool of XKCM1 is not inhibited, and only the kinetochore-bound portion of XKCM1 is affected. This reagent can be used to further dissect the function of kinetochore-bound XKCM1 in the presence of the soluble pool of the protein both in extracts and in cells.

Regulation of XKCM1 Activity: We tried several strategies to look for regulation of XKCM1 activity. To first test if XKCM1 was phosphorylated, gamma-labeled $^{32}$P-ATP was added to either interphase or mitotic extracts, and then XKCM1 protein was immunoprecipitated from the extracts. XKCM1 was phosphorylated by both interphase and mitotic extracts although the patterns of phosphorylation were different between the two extracts. Both extracts showed a doublet of labeled phosphoproteins with the interphase extract incorporating more phosphate into the lower band whereas mitotic extracts had more phosphate incorporation into the upper band. Purified baculovirus expressed protein comigrated with the faster migrating form of XKCM1 from extracts. After incubation with a mitotic Xenopus extract, the recombinant protein was now a doublet similar to that found endogenously in extracts suggesting that there is a kinase in extracts can phosphorylate XKCM1. The identification of this kinase will be an important area of further research.

Two approaches have been taken to identify the kinase that phosphorylates XKCM1 in extracts. First, the GST fusion proteins of the XKCM1 domains were added to extracts to narrow down the region where XKCM1 is phosphorylated. Only the C-terminal domain of XKCM1 was phosphorylated in the extracts. This can be used as an assay to isolate the kinase responsible. An alternative approach was to try and phosphorylate the XKCM1 domains with kinases that were thought to be involved in regulation of microtubule function in extracts. Both cdc2 kinase and MAP kinase were implicated in the control of microtubule dynamics during the transition from interphase to mitosis and could thus be likely kinases responsible for phosphorylating XKCM1. Using an *in vitro* phosphorylation reaction, neither cdc2 kinases nor MAP kinase phosphorylated any of the domains of XKCM1 suggesting that they were not involved directly in regulating XKCM1 function. CaMKII is a kinase that is activated at the metaphase/anaphase transition in Xenopus extracts. When tested against purified recombinant proteins, we found that the NT domain of XKCM1 could be highly phosphorylated by CaMKII. An analysis of the sequence indicated that there was a highly conserved CaMKII site in this NT domain which may be a physiological regulatory site. In the future, we plan to analyze the phenotypic consequences of this phosphorylation.

Analysis of XKCM1 Function in Cells: Anti-XKCM1 antibodies were microinjected into interphase PtK2 cells. Cells were incubated for 24 hrs and then fixed and stained with anti-tubulin antibodies to visualize microtubules. No differences between anti-XKCM1 injected cells and control antibody injected cells were detected. When anti-XKCM1 antibodies were injected in mitotic cells, approximately 30-50% of the injected cells

remained blocked in mitosis for greater than 2 hrs when compared to controls. These results are encouraging and will be followed up to determine where and how this block occurs.

**Recommendations in Relation to the Statement of Work:**

Below I list the aims proposed in my original statement of work. The progress achieved toward each aim is discussed as well as goals for the next year of funding.

Aim 1. Test the *in vivo* function of XKCM1 by microinjection of antibodies and expression of dominant negative forms of XKCM1 (months 1-9): We successfully generated dominant-negative forms of XKCM1 as demonstrated by our results with the GST-fusion proteins as discussed in the results and discussion. We altered our strategy to first do a thorough biochemical investigation of the XKCM1 domains and to generate a series of potential inhibitory reagents. We now have those reagents and are better prepared to explore XKCM1 function *in vivo*. The characterization of the XKCM1 domains as well as their function in spindle assembly has been presented as a talk at the American Society for Cell Biology Annual Meeting and will be presented in a poster at the Gordon Conference on motile and contractile systems. In addition, a paper describing these results is in preparation.

Aim 2. The Role of XKCM1 in *in vitro* Chromosome Movement Assays (months 10-15): In the course of our studies, we carried out a detailed mechanistic study of XKCM1 activity as presented in the results and discussion above. This study revealed some very exciting and unexpected findings in that XKCM1 does not exhibit conventional microtubule motility but rather acts to control polymerization dynamics of microtubules. Based on these findings, the *in vitro* chromosome movement assays were not appropriate to do because they required the presence of an conventional motor. The mechanistic studies were carried out in the above time frame in place of the chromosome assays. In addition the role of XKCM1 in chromosome movement is being analyzed in the context of intact spindles to also satisfy the goals of this aim. These experiments will be completed within the next 6 months. The mechanistic studies on XKCM1 were presented in a second talk at the American Society for Cell Biology Annual Meeting and will be presented in a second poster at the Gordon Conference on motile and contractile systems. In addition, a paper describing these results is in preparation.

Aim 3. Cell Cycle Regulation of XKCM1 Activity (months 15-24): These experiments were proposed for the second year of funding. However we have made significant progress already in analyzing the phosphorylation state of XKCM1 and potential kinases involved in this phosphorylation (discussed above). In addition we have identified a potential regulatory site in the kinetochore targeting domain of XKCM1. The function of this phosphorylation will be explored as described in the original proposal.

Aim 4. Structural Analysis of XKCM1 (months 1-24): These experiments are part of a long term project in determining the 3-dimensional structure of XKCM1. We have been successful in our scale-up of the XKCM1 purification to a scale sufficient for crystallization. We have set up several trays for crystallization but have not yet obtained any crystals. In addition, our biochemical analysis has pointed to several smaller constructs that should be tried for expression and purification. The generation of these constructs is in progress.

## CONCLUSIONS:

We have been studying a kinesin-related protein called XKCM1 that possesses a unique activity in that it regulates polymerization dynamics of microtubules. We have demonstrated that XKCM1 is required for spindle assembly in vitro using Xenopus egg extracts and speculate that it will have this activity in vivo as well. A detailed biochemical and mechanistic study revealed that XKCM1 activity is very novel for a member of the kinesin-related protein family; it has the ability to depolymerize microtubules rather than translocate along them. This was a very novel and unexpected finding and has strong implications in understanding the biochemical mechanism of this class of protein as well as understanding the role of microtubule dynamics in cellular function. We have shown that inhibition of a specific pool of XKCM1, that which is bound to kinetochores causes a misalignment of chromosomes on the spindle. This data provides the first molecular handle on a protein that can couple microtubule dynamics to chromosome movement, a molecule that has long been sought after by cell biologists studying mitosis. Finally, we have begun an analysis of the regulation of XKCM1 activity as well as set up reagents to dissect XKCM1 function in living cells. These are high priority experiments as this protein will clearly play an important role in spindle function in cells.

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